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Review

Newcastle disease: Evolution of genotypes and the related diagnostic challenges

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ABSTRACT

Since the discovery of Newcastle disease virus (NDV) in 1926, nine genotypes of class I viruses and ten of class II have been identified, representing a diverse and continually evolving group of viruses. The emergence of new virulent genotypes from global epizootics and the year-to-year changes observed in the genomic sequence of NDV of low and high virulence implies that distinct genotypes of NDV are simultaneously evolving at different geographic locations across the globe. This vast genomic diversity may be favored by the large variety of avian species susceptible to NDV infection and by the availability of highly mobile wild bird reservoirs. The genomic diversity of NDV increases the possibility of diagnostic failures, resulting in unidentified infections. Constant epidemiological surveillance and pro-active characterization of circulating strains are needed to ensure that the immunological and PCR reagents are effective in identifying NDV circulating worldwide. For example, in the United States, the widely used real-time reverse transcription polymerase chain reaction (RRT-PCR) matrix gene assay for the identification of NDV often fails to detect low virulence APMV-1 from waterfowl, while the RRT-PCR fusion gene assay, used to identify virulent isolates, often fails to detect certain virulent NDV genotypes. A new matrix-polymerase multiplex test that detects most of the viruses currently circulating worldwide and a modified fusion test for the identification of virulent pigeon viruses circulating in the U.S. and Europe have recently been developed. For newly isolated viruses with unknown sequences, recently developed random priming sequencing methods need to be incorporated into the diagnostic arsenal. In addition, the current system of classifying NDV into genotypes or lineages is inadequate. Here, we review the molecular epidemiology and recent diagnostic problems related to viral evolution of NDV and explain why a new system, based on objective criteria, is needed to categorize genotypes.

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Contents

1. Newcastle disease (ND)

Newcastle disease (ND) results from infections with virulent Newcastle disease viruses (NDV), having intracerebral pathogenicity indices (ICPI) of ≥0.7 in day-old chickens (*Gallus gallus*) and/or

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having multiple basic amino acids (at least three arginine (R) or lysine (K) residues) at the C-terminus of the fusion protein cleavage site, starting at position 113, along with a phenylalanine at position 117 (OIE, 2009). NDV potentially infects most species of birds, and for susceptible poultry it is highly contagious and usually fatal (Alexander, 1988). This disease is one of the most important infectious diseases of poultry because of its worldwide distribution and the potential for devastating loses. It occurs on at least six of the seven continents of the world and is enzootic in many countries. In the United States (U.S.) virulent NDV (vNDV) strains are classified as select agents. These strains are not endemic in poultry and disease caused by them is often referred to as exotic Newcastle disease (END) (USDA, 2006). Worldwide, outbreaks of vNDV are a constant threat to poultry with clinical infection confirmed in 2008 from the Dominican Republic, Belize, Peru, Finland, Germany and Japan and likely other outbreaks not reported from countries throughout Africa, and Asia with endemic vNDV.

Clinical manifestation of ND in chickens varies significantly among isolates. Infection with virulent viruses causes three well-defined clinical forms: (1) viscerotropic velogenic, which is characterized by acute lethal infections, usually with hemorrhagic lesions in the intestines of dead birds, (2) neurotropic velogenic, which is characterized by high mortality following respiratory and neurological disease, but where gut lesions are usually absent, and (3) mesogenic, which causes respiratory and neurological signs, but low mortality. The presence of virulent strains (velogenic and mesogenic strains) in poultry requires monitoring and control measures even in countries where they are endemic because the existence of the virus severely impacts commercial productivity and the international trade in poultry and poultry products. Low virulence NDV (loNDV) are often referred to as lentogenic and are endemic in the U.S. In addition to being classified as being of low virulence, causing mild infections of the respiratory tract, lentogens can also be classified as asymptomatic enteric in which replication appears to be primarily in the gut without respiratory signs.

2. Newcastle disease virus (NDV)

Newcastle disease virus, also known as avian paramyxovirus serotype-1 (APMV-1), a member of the genus Avulavirus within the Paramyxoviridae family (Fauquet and Fargette, 2005; Mayo, 2002), is a negative-sense, single stranded, non-segmented, enveloped RNA virus (Alexander and Senne, 2008). The NDV genome is composed of six genes and encodes their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L) (Chambers et al., 1986). RNA editing of the P protein produces additional non-structural proteins V and possibly W (Chambers and Samson, 1982; Collins et al., 1982; Locke et al., 2000; Mebatsion et al., 2001; Steward et al., 1993). While the virulence of NDV is dependent on multiple genes, the fusion protein cleavage site is the critical site responsible for major changes in virulence (de Leeuw et al., 2005; Peeters et al., 1999). The less virulent viruses (loNDV) have fewer basic amino acids at this site and a leucine instead of a phenylalanine at position 117 (Alexander and Senne, 2008). Using reverse genetic techniques it has been shown that the phenylalanine at position 117 and basic amino acids surrounding Q 114 are necessary for virulence (de Leeuw et al., 2003; Glickman et al., 1988).

3. Newcastle disease virus classification

Different genotypes of APMV-1 circulate in different parts of the world. Although all NDV are members of APMV-1, antigenic and genetic diversity is recognized (Aldous et al., 2003; Alexander et al., 1997; Kim et al., 2007a). Two different systems of classifying NDV are currently utilized worldwide with no consensus as to which is more appropriate. A system suggested by Aldous groups NDV into six lineages and 13 sublineages and later, three additional sublineages were added (Snoeck et al., 2009; Aldous et al., 2003). A second system classifies NDV into two major divisions represented by class I and class II, with class I being further divided into nine genotypes and class II into ten when comparing the sequences isolated over time (Fig. 1A and B) (Ballagi-Pordany et al., 1996; Czegledi et al., 2006; Kim et al., 2007b). Both systems of classification are based on similar genomic information therefore discrepancies between the two classification systems are nominal. Fig. 1B, showing class II viruses, illustrates the most significant differences between the two systems. For example, genotypes III, IV, V and VIII are grouped in the Aldous system to create the very large lineage 3. Problems with both nomenclature systems are illustrated in Table 1, which lists the estimates of the average evolutionary divergence among genotypes. The overall distances between genotypes range widely from to 0.041 (between III and IV) to 0.181 (between II and VII) (Table 1). In the case of genotype VI the distances between sub-genotypes VIc and VIf (0.061) or between VIf and VIb (0.054) are larger than the distances between genotype III and IV (0.041) suggesting that genotype VI may need further separation. Estimates of average evolutionary divergence over sequence pairs within genotypes demonstrate the existence of a large variation of distances among viruses within each genotype (Table 2). For example, a distance within genotype IX is 0.004 and it is 0.085 for viruses within genotype III. These wide differences in distances separating genotypes and the lack of any

Table 1
Estimates of evolutionary divergence over sequence pairs between genotypes.

	Genotype									
	II	IIwfl	III	IV	V	VI	VII	VIII	VIb	VIc
I	0.103	0.070	0.067	0.080	0.131	0.115	0.133	0.114		
II		0.111	0.124	0.153	0.176	0.164	0.181	0.166		
IIwfl			0.100	0.122	0.160	0.144	0.147	0.157		
III				0.041	0.100	0.099	0.108	0.090		
IV					0.089	0.082	0.092	0.087		
V						0.075	0.084	0.078		
VI							0.065	0.075		
VII								0.079		
VIc									0.047	
VIf									0.054	0.061

The number of base substitutions per site is shown for genotypes of class II viruses. All results are based on the pairwise analysis of 1003 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA4 (Tamura et al., 2004, 2007). Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 372 positions in the final dataset. wfl = waterfowl isolates also called genotype IIa.

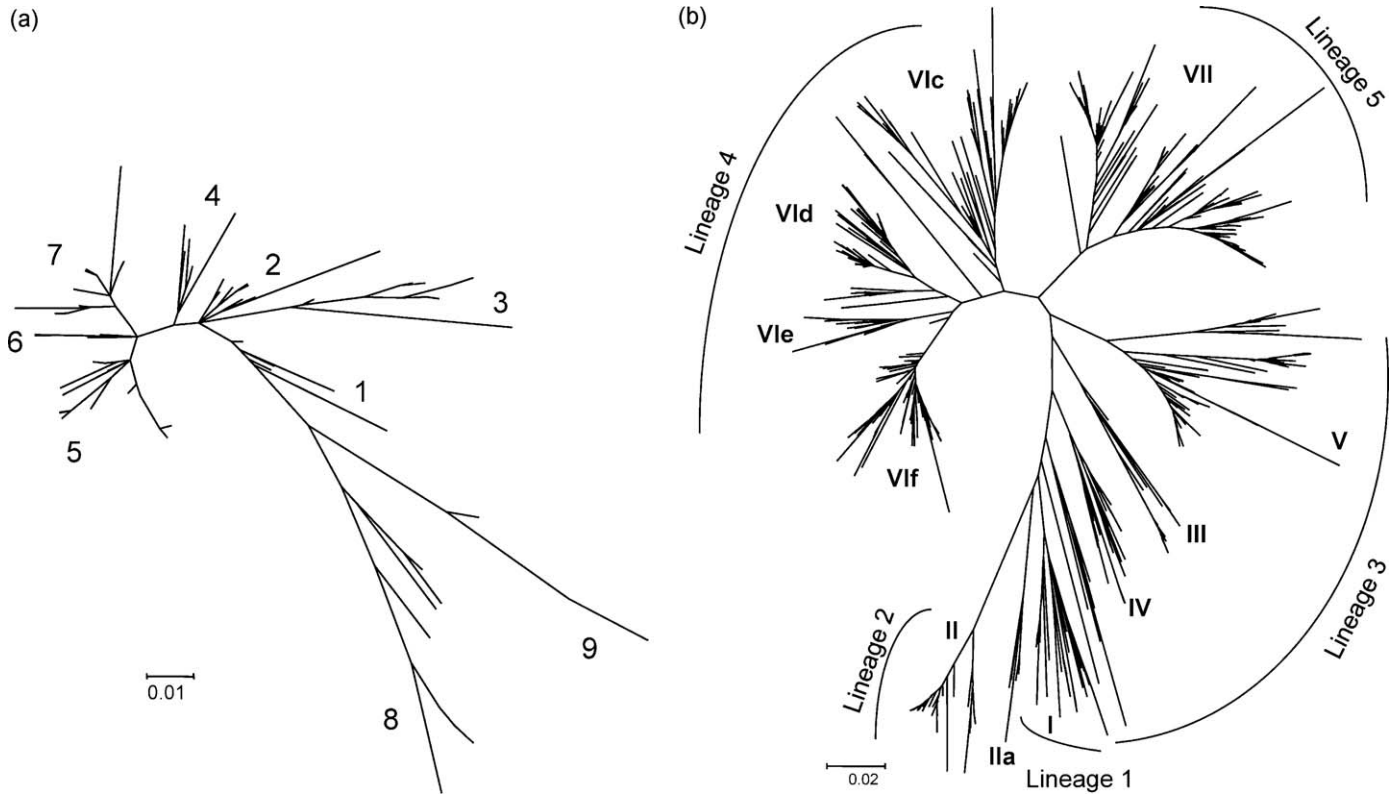

Fig. 1. Phylogenetic trees of Newcastle disease viruses. Tree construction was done using the Neighbor Joining method with the maximum composite likelihood substitution model for the 372 bp region encoding the amino terminal end of the fusion protein (Tamura et al., 2004, 2007). (a) Class I viruses (n = 216). (b) Class II viruses (n = 956) and genotypes are labeled with arabic numberals for (a) and roman numerals for (b). The sub-genotypes of genotype VI are included. Genotypes VIII, IX, and X for class II viruses are not included.

other additional criteria (e.g. antigenic distances) to separate genotypes suggest the need for a scientific conference to discuss and establish the objective standards that would be needed to create a widely recognized and consistent classification.

In this review, we will utilize the second classification system. APMV-1 viruses have at least three genome lengths; 15,186, 15,192 and 15,198 nucleotides (Czegledi et al., 2006). Class I viruses are avirulent in chickens (except for one known virulent virus) and historically have been recovered from waterfowl (Family Anatidae) and shorebirds (Alexander et al., 1992; Kim et al., 2007a). Class I viruses have the longest of the APMV-1 genomes at 15,198 nucleotides (Czegledi et al., 2006). These viruses comprise at least nine (1–9) genotypes that may be distributed worldwide in wild birds (Kim et al., 2007a) and are frequently isolated in live bird market (LBM) samples (Fig. 1A) (Kim et al., 2007a,b).

Table 2
Estimates of evolutionary divergence over sequence pairs within genotypes.

Genotype	Distance
I	0.079
II	0.027
IIwfl	0.029
III	0.085
IV	0.057
V	0.056
VI	0.076
VII	0.071
VIII	0.068
IX	0.004

The number of base substitutions per site is shown for genotypes of class II viruses. All results are based on the pairwise analysis of 1003 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA4 (Tamura et al., 2004, 2007). Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 372 positions in the final dataset. wfl = waterfowl isolates also called genotype IIa.

Class II viruses have been studied in more detail and comprise ten (I–X) genotypes. The genotypes that are considered "early" (1930–1960) I, II, III, IV and IX contain 15,186 nucleotides (Czegledi et al., 2006). Viruses that emerged "late" (after 1960), V, VI, VII, VIII, and X contain 15,192 nucleotides. Except for recent vNDV responsible for the 1998–2000 Australian outbreak, all other known class II, genotype I viruses are of low virulence and some are often used as live vaccines (chicken/Australia/QV4/1966 and chicken/N. Ireland/Ulster/1967). Class II, genotype II includes viruses of low virulence that are used as vaccine viruses worldwide, such as LaSota, B1 and VG/GA. It also includes the neurotropic virulent chicken/U.S. (TX) GB/1948 (TXGB) isolate, which was isolated in 1948 and is used in the USA as a challenge to show efficacy of ND commercial vaccines before production. In the U.S., in the 1940s and 1950s the above-mentioned vaccines were highly effective against the circulating neurotropic vNDV, such as TX/GB and Kansas-Manhattan strains. In fact, virulent neurotropic viruses of genotype II no longer circulate in the U.S. Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and in Zimbabwe in 1990 (Yu et al., 2001). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi et al., 2006).

Genotypes V, VI, VII, and VIII are the predominant genotypes circulating worldwide and contain only virulent viruses. Genotype

V viruses emerged in South and Central America in 1970 and caused outbreaks in Europe that same year (Ballagi-Pordany et al., 1996). These viruses also caused outbreaks in North America in Florida (1971, 1993) and California (1971, 2002) (Wise et al., 2004a) and are still circulating in Mexico (Perozo et al., 2008). Genotype VI emerged in the 1960s and continued to circulate as the predominant genotype in Asia until 1985 when genotype VII became more common (Mase et al., 2002). Genotype VI is further divided into sub-genotypes VIa through VIg with VIb being commonly isolated from pigeons.

Genotype VII was initially divided in to two sub-genotypes: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous et al., 2003). The two sub-genotypes of VII are further divided into VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa (Bogoyavlenskiy et al., 2009; Wang et al., 2006), and VIIf, g, and h, which represent African isolates (Snoeck et al., 2009). Genotype VIII viruses have been circulating in South Africa since 1960s (Abolnik et al., 2004a) and continue to circulate in Southeast Asia. Genotype IX is a unique group that includes the first virulent outbreak virus from China from 1948 and members of this genotype continue to occasionally be isolated in China (Wang et al., 2006). Genotype X viruses have been isolated exclusively from Taiwan in 1969 and 1981 (Tsai et al., 2004). Fig. 2 illustrates the yearly distribution of genotypes separated by decade based on sequences reported to GenBank. It indicates that multiple genotypes are circulating simultaneously worldwide and reveal an increase in the number of (reported) genotypes circulating at the present time. With the exception of genotype IV, which has not been reported to GenBank since 1989, viruses from most genotypes still continue to circulate at the present time.

4. The evolution of NDV of low virulence

Very little is known about the evolution of NDV of low virulence (loNDV) as most NDV research has been performed on virulent isolates. Globally, loNDV from all genotypes of class I and from genotypes I and II of class II are commonly isolated from domestic poultry and wild bird species (Huovilainen et al., 2001; Jorgensen et al., 1999; King and Seal, 1997; Marin et al., 1996; Rosenberger et al., 1975; Seal et al., 2005; Takakuwa et al., 1998). In the U.S., where vNDV are not endemic in poultry, the primary reason for vaccination is to protect against economic losses from respiratory disease caused by endemic loNDV (Miller et al., 2009a). In addition, if a poultry flock is not uniformly vaccinated with a live NDV vaccine or if another vaccine interferes with the NDV vaccine, the birds sub-optimally vaccinated for ND may "break" with respiratory disease days later. These vaccine reactions are called "rolling reactions." As the virus passes from bird to bird, the respiratory disease seen may be more severe than that originally seen with the initial application of the vaccine (J.A. Smith, personal communication, 04/2009). Although viruses involved in rolling reactions are presumed to be of vaccinal origin, without the genomic characterization of these isolates, the possible involvement of loNDV from wild birds in rolling reactions cannot be discounted.

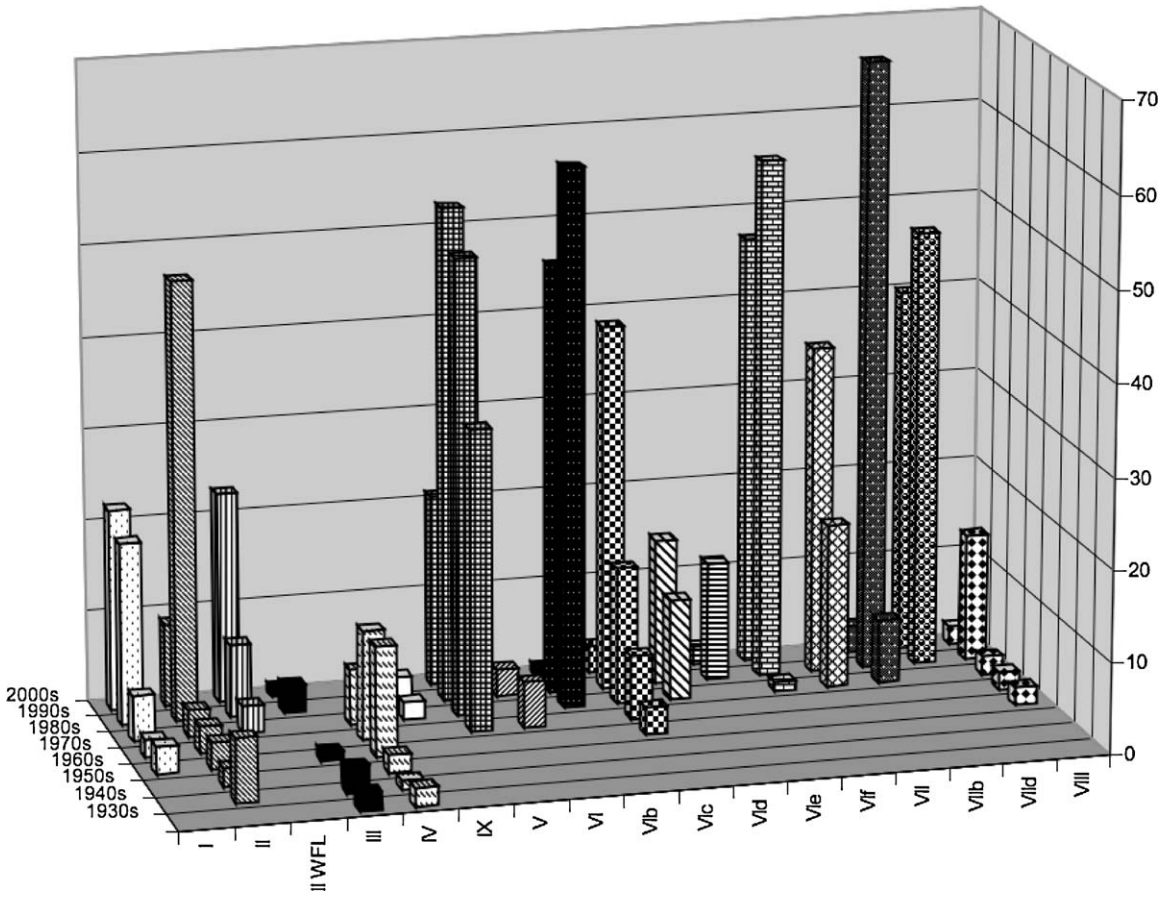

Fig. 2. Class II viruses used in Fig. 1B were sorted by date of isolation and the number of viruses reported to GenBank for each of the genotypes per decade was graphically represented. *X* axis represent genotypes, *Y* represent decades and *Z* represent the number of isolates. The number of virus isolates reported for each genotype per decade: G I (1962–2004) $n = 52$; G II (1945–2001) $n = 78$; G III (1930–2002) $n = 14$; G IV (1933–1989) $n = 37$; G V (1970–2003) $n = 158$; G VI (1968–2002) $n = 343$; G VII (1981–2002) $n = 250$; G VIII (1965–2000) $n = 24$; G IX (1985–1997) $n = 11$. Genotype X is not included.

Wild waterfowl and shorebirds (WS) are infected with a large and diverse group of avirulent viruses that normally do not produce any clinical signs in poultry. A recent phylogenetic evaluation of avirulent NDV isolates from U.S. LBM and WS found that about 70% of these viruses belong to class I and 30% to class II (Kim et al., 2007a). Almost half of the class I viruses came from the Mallard duck (*Anas platyrhynchos*). In this single species, six of the nine class I genotypes were represented, suggesting that certain species are highly susceptible to NDV infection and likely to be reservoirs. Wild WS were carrier of class II viruses however no virulent virus was present. These class II viruses grouped into genotype I and two new sub-genotypes: IIa and Ia. The Ia viruses were related to viruses endemic to Australia, including the virulent virus isolated from the 1998 Australian outbreak. The IIa isolates grouped with viruses previously isolated from Argentina (AY727881, duck and AY727882, swan) and Spain (AY175642, chicken). None of the WS viruses clustered into genotype II with the common vaccine viruses, B1 and LaSota. This is surprising considering the amount of live virus that is applied annually to poultry in the U.S and worldwide.

As previously described with virulent class II viruses diversity was observed at the antigenic level by comparing monoclonal antibody (mAb) binding patterns among different ND isolates (Alexander et al., 1997). For example, when a panel of nine mAb was tested against 58 U.S. viruses isolated from Anatidae species from 1986 to 1999, seven different binding patterns were revealed (Kim et al., 2007a). Considering that in this study, only avian WS species from the orders Anseriformes and Charadriiformes were studied and that NDV has been documented to infect at least 241 different bird species (Kaleta and Baldeuf, 1988), the existence of NDV with different mAb binding patterns in other birds species is plausible. The broad phylogenetic diversity of NDV discovered in this limited wild bird population emphasized the potential for more genotypes yet to be discovered.

Class I viruses, in general, are viruses of wild birds. The discovery of a large number of class I ND viruses from LBMs in the U.S. and Asia, and the existence of genetically related viruses in wild birds suggests that epidemiological connections exist between these populations. A close phylogenetic relationship was found between some class I LBM isolates and WS isolates collected in the U.S. from 1986 through 2005 (Kim et al., 2007a). Class I viruses were also isolated from LBM domestic poultry in Hong Kong (HK) from 2003 to 2005, suggesting that these viruses were circulating in LBM birds and wild birds worldwide (Kim et al., 2007b). The identification of a class II genotype Ia virus from a 12-week-old domestic turkey in Ontario in 2003 (AY289194), which clustered closely with U.S. shorebird isolates from New Jersey in 2001 (EF564885, EF564816, EF564888–EF564889), may indicate that these viruses occasionally transmit between wild bird and domestic poultry populations. Class II genotype Ia and I viruses have also been isolated previously from chickens at LBM in the U.S. (AY130861, AY626268) (Kommers et al., 2003; Seal et al., 2005).

While NDV isolated from WS are predicted to exhibit low virulence phenotypes, concern exists regarding possible genetic changes from loNDV to vNDV upon replication in poultry. At least one virulent class I virus has been identified as the etiology of an outbreak in the past (Alexander et al., 1992). Virulent viruses of similar genotypes to those found in WS and at LBM have previously been recovered (Alexander et al., 1992; Gould et al., 2001). The 1990 Ireland outbreak was caused by a virulent form of a class I virus (IECK90187/1990; accession AY135757), and the Australian outbreak of 1998–2000 was caused by a virulent class II genotype Ia virus. It was theorized that low virulence viruses circulating in and among the waterfowl of Ireland were transmitted to poultry and mutated to form quasi-species from which a virulent class I virus arose (Collins et al., 1993). Genomic analysis of the class II genotype Ia viruses from the Australian outbreak provided additional evidence that low virulence viruses have the potential to become virulent over time (Gould et al., 2001), and demonstrated that a change in the cleavage site of the fusion protein of the native virus resulted in increased virulence (Gould et al., 2003). Virulent isolates from outbreaks in Australia were shown to be genetically similar to the viruses of low virulence that were known to be previously circulating in the country (Kattenbelt et al., 2006). These endemic low virulence viruses required only two point mutations to become virulent (Westbury, 2001). In addition to field conditions, some loNDV have shown to have the capacity to become virulent under experimental conditions (de Leeuw et al., 2003; Shengqing et al., 2002; Zanetti et al., 2008). Three factors that may increase the risk of an outbreak and predict the need to conduct studies on the evolutionary mechanism affecting NDV genomes are: (1) only a few nucleotide changes in the fusion gene are sufficient to change NDV from low virulence to high virulence, (2) there are large and highly mobile reservoirs of low virulence viruses in nature that may come into contact with poultry, and (3) billions of doses of low virulence live vaccine virus are inoculated into poultry annually, thereby likely releasing the vaccine virus into the environment. For the purposes of disease prevention it would be ideal to be able to predict the potential of each genotype of low virulence NDV to mutate into a virulent form.

Recently, a comprehensive dataset of NDV genome sequences was evaluated using bioinformatics to characterize the evolutionary forces affecting NDV genomes and it was found that there was no evidence of positive selective pressures at the fusion protein cleavage site (Miller et al., 2009a,b). This study demonstrated negative or neutral selective pressure at the fusion protein cleavage site for both vNDV and loNDV. Specifically, regardless of the genotype or the virulence of the virus, the cleavage site motif was highly conserved, implying that changes in virulence were unlikely to occur frequently. Interestingly, the complete coding sequence of the fusion gene of diverse isolates and the complete genomes of viruses from wild birds displayed higher yearly rates of change in virulent viruses than in viruses of low virulence, indicating that higher virulence may confer some other positive selective advantages to NDV (Miller et al., 2009b).

5. Virulent NDV and the role of ND vaccines on their evolution

Although the most likely reservoir of vNDV is the vaccinated poultry population there is evidence that wild birds may represent natural reservoirs of mesogenic viruses (Aldous et al., 2007; Czegledi et al., 2006). Phylogenetically related vNDV of genotype V have been isolated from double-crested cormorants (*Phalacrocorax auritus*) from 1975 through 2008 and they have been implicated in earlier ND outbreaks (Allison et al., 2005; Blaxland, 1951; Heckert et al., 1996). Virulent pigeon paramyxovirus-1 (PPMV-1) isolates, which are clinically neurotropic in chickens, were first isolated in 1981 in pigeons (*Columba livia*), and continue to circulate in feral birds of the Columbidae family worldwide (Kaleta et al., 1985; Kim et al., 2008a; Mase et al., 2009; OIE, 2003). Although an earlier isolation of viscerotropic vNDV was documented in pigeons (Pearson and McCann, 1975), typical pigeon isolates are variants from genotype VIb, that cause neurological signs, have specific monoclonal antibody binding patterns, and often do not hemagglutinate chicken red blood cells well (Ujvari et al., 2003). Other virulent viruses have been isolated sporadically from imported tropical bird species but the existence of a reservoir in these other species remains to be confirmed. In fact, it has been shown that some wild birds were not natural reservoirs of vNDV, but infected at quarantine stations prior to export (Kaleta and Baldeuf, 1988).

The vNDV isolated from cormorants and pigeons are considered mesogenic because their ICPI values in chickens vary from >0.7 to

<1.5 and they do not usually cause significant disease in poultry. However, recently the U.S. designated all NDV with ICPI values >0.7 or containing an amino acid sequence consistent with virulent strains of NDV as virulent and classified them as select agents, to follow the World Organization for Animal Health (OIE) and the European Union standards (EU) (APHIS, 2008; OIE, 2004). Although cormorant and pigeon viruses continue to evolve and display year-to-year genomic changes, no significant changes in virulence have been observed in samples obtained from wild birds (Weingartl et al., 2003).

The almost exclusive predominance of low virulence class I and mesogenic viruses of class II, genotypes V or VI in cormorants and pigeons, in contrast to the prevalence of viscerotropic vNDV (class II, genotypes V–X) in vaccinated poultry (Czegledi et al., 2006), suggests that the immune pressure from vaccination may be selecting variant forms of vNDV. This puzzling persistence of vNDV in poultry despite intensive vaccination efforts has been a recurrent phenomenon in endemic countries of Asia, Africa and Central America (Alexander, 2001; Czegledi et al., 2006). It is possible that the combined characteristics of current poultry production systems including: (1) host genetic homogeneity (with few host adaptive bottlenecks), (2) high density rearing (allowing close animal-to-animal contact, and favoring transmission of highly virulent virus over milder forms), and (3) intensive vaccination programs (which provide selective immune pressures and may be executed improperly in developing countries), may contribute to the evolution of virulent viruses (Higgins and Shortridge, 1988). However, to date, well-controlled studies have not been performed to demonstrate a role of vaccination on the evolution of virulent NDV.

Large phylogenetic and antigenic distances between vaccines and current circulating virulent strains may facilitate the evolution of virulent NDV (Miller et al., 2007). Many have demonstrated that current vaccines prevent disease but cannot stop viral shedding (Kapczynski and King, 2005; Miller, 2009; Utterback and Schwartz, 1973). In addition, evidence that using genotype-matched vaccines can significantly reduce viral shedding is emerging. More recently two antigenically matched vaccines have demonstrated increased capacity to prevent viral shedding of viruses of genotype VII and V, respectively (Hu et al., 2009; Miller, 2009).

Existence of field-isolated variants that escape vaccination has been presented (Cho et al., 2008a). NDV variants were isolated at multiple locations and time points in Korea with an increase in the frequency of isolation from 21% in 2002 to 85.7% in 2003–2006. Unfortunately, as the total number of isolates analyzed was small, the epidemiological significance is uncertain (56 total samples). These authors further identified one variant epitope in the HN gene and developed a recombinant vaccine utilizing the variant virus HN gene in a LaSota strain backbone (Cho et al., 2008a). A killed vaccine virus containing a matched HN gene was used and demonstrated to be equally protective when compared to commercial vaccines against morbidity and mortality. However, their recombinant vaccine, homologous to the challenge virus, was more effective in preventing decreases in egg production. Unfortunately, no evaluation of viral shedding was done in this study.

Genetic variants may also be occurring in Mexico. NDV was isolated for the first time in Mexico in 1946, and the last detailed report of a field outbreak caused by a highly virulent strain dates from the year 2000, when 13.6 million birds were slaughtered and 93 farms quarantined (OIE, 2003). After these outbreaks extensive vaccination programs were implemented in the affected areas. A phylogenetic comparison of the complete coding region of the fusion gene from recent Mexican viruses to previously published NDV from other known class II genotypes indicated that all the viruses isolated in Mexico between 1998 and 2006 grouped with the class II, genotype V viruses (Perozo et al., 2008). However, a clear phylogenetic separation of two groups of viruses analyzed in this study into either an early (1998–2001) or a recent (2004–2006) group, along with the ICPI and mean death time (MDT) results (traditionally, used to evaluate the pathogenicity of any new NDV isolate), suggested that some distinct selective pressure on the 1998–2001 viruses may have led to the appearance of the new group. The 1998–2001 isolates showed a 98% very high percent identity with other Mexican viruses reported between 1996 and 2001; but these were only 93–94% identical to recent isolates. Unfortunately, no studies were performed to characterize the antigenic differences between both groups of viruses.

Evidence that NDV vaccines may not be very effective under specific conditions is provided by field observations from Mexico (www.sagarpa.gob.mx). In addition, to the continued vNDV outbreak in backyard flocks, a drop in egg production levels in well-vaccinated birds displaying high levels of antibody to NDV has been observed since 2005 (Castilla and colleagues, 2009 ANECA abstract), similar to the egg production problems described in South Korea (Cho et al., 2008b). With the absence of clinical signs, the presence of vNDV has been confirmed by viral isolation and histopathology.

Evidence that current ND vaccines fail to protect against morbidity and mortality caused by new variants from genotype VII, is more controversial. One report, based on cross protection experiment suggests that at least two recent Chinese isolates from 2001 and 2003 are antigenic variants (Qin et al., 2008). In this report, 50, 40 and 10 percent of the birds vaccinated with a live LaSota vaccine displayed disease symptoms after being challenged with possible antigenic variants, in comparison to all birds being without clinical disease after being challenged with the viruses that were not identified as variants. However, evidence of the immune status (hemagglutination-inhibition assay or ELISA antibodies) of the vaccinated animals prior to and after challenge was not presented. In a similar experiment, in which no corroborating antibody data was presented, a 1996 isolate was reported as an antigenic variant (Yu et al., 2001). In contrast to these studies, two manuscripts demonstrate that current vaccines have good protective efficacy for morbidity and mortality against viruses currently circulating in Asia (Jeon et al., 2008; Liu et al., 2003). In one study, chickens vaccinated with either a live or a killed oil-emulsion LaSota (genotype II) vaccine, were fully protected against heterologous challenge strains of genotypes VIg, VIb, VIId and IX (Liu et al., 2003). Similarly, the other study demonstrated protective efficacy of two commercial vaccine strains in SPF chickens against two virulent challenge viruses of genotype VII (Jeon et al., 2008). Despite the controversy, enough evidence exists to suggest that NDV variants may be created in poultry as a result of suboptimal vaccination.

6. Diagnostic challenges

6.1. Detection of all NDV

Newcastle disease is generally diagnosed by isolation of NDV in SPF embryonating chicken eggs (ECE), by serology using the hemagglutination-inhibition (HI) test, or by real-time RT-PCR (RRT-PCR). All NDV isolates are known to replicate in ECE and the MDT to kill the embryo varies depending on the virulence of the virus. The HI test is used to identify a virus as NDV. Monoclonal antibody (mAb) testing can further be used to characterize NDV. While no single mAb can determine the virulence of NDV, a panel of mAb can be used to characterize differences, for example viruses of loNDV from PPMV-1. In addition, the use of mAb assays for rapid characterization of these viruses is not optimal for class I viruses as most of the mAb were developed and optimized to recognize class II viruses and fail to recognize viruses of class I (Collins et al., 1998;

Kim et al., 2007b). Because mAbs are directed against single epitopes, individually their ability to detect a broad spectrum of viruses is often limited. Other serologic assays such as ELISA are also used in diagnostic laboratories to assess antibody response following vaccination, but have limited value in surveillance and diagnosis because of the almost universal use of vaccines in domestic poultry.

Following identification, pathotyping of isolates is required to determine virulence characteristics. The methods used to pathotype newly isolated strains of the virus include the intracerebral pathogenicity index (ICPI) test, MDT and determining the amino acid motif at the cleavage site of the fusion protein. As pathotyping tests are time consuming and expensive and serological tests are complicated by the universal use of live-virus vaccines in poultry, rapid nucleic acid based assays have been developed. Multiple single-tube, sensitive, rapid real-time reverse transcription polymerase chain reaction (RRT-PCR) assays have been developed in the last decade around the world to detect the viruses circulating in those locations (Antal et al., 2007; Fuller et al., 2009; Pham et al., 2005; Tan et al., 2004; Wise et al., 2004b). In addition, other techniques and variations of RT-PCR increase the number of techniques available to detect NDV (Farkas et al., 2007; Wang et al., 2008).

In the United States, two different RRT-PCR assays have been used extensively and field validated to identify APMV-1 viruses (matrix gene assay) and to differentiate virulence (fusion gene assay), respectively (Wise et al., 2004b). They were developed to detect class II NDV in diagnostic clinical samples from birds using fluorogenic probes and were validated by the U.S. Department of Agriculture in response to the 2002 END outbreak in the state of California, which was contained in 2003 (OIE, 2004). Depending on assay conditions, these RRT-PCR tests can be equally or more sensitive than virus isolation, but are always faster than virus isolation (VI) and have been adopted as the standard method for surveillance in the U.S. Although these assays were used and shown to be effective during the California outbreak for screening flocks (Crossley et al., 2005), at the time of the assay's development and testing, not all clinical samples that were virus isolation (VI) positive were detected by the assay, which led the developers to reiterate that the assay should be used for screening flocks and not for individual samples.

6.2. RRT-PCR to detect all NDV

The primers and probe for the M-gene assay were designed to detect the highly conserved matrix (M) gene of NDV and, as such, will detect most NDV genotypes of class II, regardless of pathotype. However, due to the heterogeneous genetic nature of this virus, class I viruses tested often fail to be detected (Kim et al., 2007a). More recently, the M-gene assay was used to characterize hemagglutination-positive surveillance samples from waterfowl and shorebirds. It was found that approximately 70% of these isolates that corresponded to class I viruses were poorly or not detected (Kim et al., 2007a,b). Evaluation of the nucleotide sequence alignment of the M-gene assay probe site of class I and II viruses revealed a high number of mismatches between the two classes, and this is likely the reason that the class I viruses escape detection by this assay.

A new matrix-polymerase multiplex RRT-PCR was developed for the detection of a broad range of class I and II NDV isolates (Kim et al., 2008b). A conserved region from the polymerase (L) gene of class I NDV genomes was identified and used in the design and evaluation of a multiplex RRT-PCR assay (L-TET) that identifies a broad range of NDV. This L-TET assay, which demonstrated a ten-fold increase in sensitivity over the previously reported L-gene assay, identifies previously missed class I isolates, and works in conjunction with the existing M-gene assay using the same protocol (Kim et al., 2007b). Differential detection of mixed class I and II viruses down to 100 fg was possible because L-TET uses an alternate fluorophore from the M-gene assay. The multiplexed assay was capable of detecting a broad range of class I and II NDV ($n = 108$) genotypes 1–2, 4–9 and ($n = 32$) genotypes I to VII, respectively, all of which were correctly identified by both the single- and multiplex formats.

6.3. RRT-PCR to detect virulent isolates

The ability to detect virulent viruses quickly is key to containing an outbreak. The F gene probe created specifically to detect virulent NDV from field swabs during the outbreak of 2002 in the U.S. is widely used because it was field validated. The primers and probe match the genome of the California 2002 virus closely, however the test was and was not intended to detect vNDV worldwide (Wise et al., 2004b). During the initial testing, this assay was able to identify a wide range of isolates from different worldwide locations, except for one Dove/It virus collected from a collard dove (*Streptopelia decaocto*) in Verona, Italy in 2000 (AF520965) (Terregino et al., 2003). Dove/It is phylogenetically related to other pigeon adapted NDV and has a unique cleavage site motif with a lysine (K) replacing a glutamine (Q) at residue 114 that has been reported in other NDV isolated from pigeons in Finland and Germany (Huovilainen et al., 2001; Oberdorfer and Werner, 1998). The isolates with this cleavage site motif represent a distinct subset of PPMV-1 NDV (Aldous et al., 2004). Because all of the other PPMV-1 tested had been detected by the F-gene assay, further studies were performed to determine why Dove/It escaped detection. Sequence analysis of the Dove/It isolate identified four nucleotide differences with the F gene probe sequence corresponding to positions 1, 6, 13, and 14 of the probe (Kim et al., 2006). Four nucleotide differences between the Dove/It isolate genome and the fusion test probe appeared to be responsible for the test failure.

More recently, NDV isolates recovered from dead birds from the order of Columbiformes in Rhode Island and eastern Texas between 2000 and 2007 were positively identified as NDV by the RRT-PCR M-gene assay (Kim et al., 2008a). Monoclonal antibody patterns of nine of the 15 isolates showed the typical pattern seen with PPMV-1 and due to their ICPI values being greater than 0.7 or by their fusion cleavage sites having the ^{112}RRKKRF^{117} motif, all 15 isolates were defined as virulent. All of these isolates were negative by the F-gene assay. A new probe, designed to account for the three mismatches in the Dove/It isolate probe site previously identified, was able to identify these virulent PPMV-1 (Kim et al., 2008a).

Pigeon paramyxoviruses are distributed worldwide. Although many countries maintain compulsory vaccination of racing pigeons, there is no form of disease control in wild pigeons, which frequently have contact with backyard and free-range poultry (Alexander et al., 1984, 1985; Toro et al., 2005). As virulent PPMV-1 isolates continue to circulate and evolve in pigeons and doves worldwide (Abolnik et al., 2008; Kim et al., 2008a; Liu et al., 2007), outbreaks of PPMV-1 in chickens are feasible, as they have occurred in the past (Abolnik et al., 2004b; Alexander et al., 1984). It is imperative that diagnostic laboratories using the USDA validated or other fusion protein based PCR assays continue to monitor genomic changes and re-design alternate primers and probes to prevent the failure of detection of PPMV-1. Alternatively, the use of virus isolation in eggs in conjunction with the PCR assays will identify these isolates with embryo mortality.

6.4. Random priming methods

The use of random sequencing methods for diagnostic purposes is based on the following premises. The high capacity for mutation in RNA viruses (genetic drift) and the large diversity of NDV genotypes often makes it difficult to predict the genetic composition of new

isolates. Efforts to identify new isolates have focused on the development of DNA microarrays, real-time PCR, or rapid sequencing of RRT-PCR products (Hoffmann et al., 2007; Kiss et al., 2006; Romano et al., 1996; Spackman et al., 2002; Townsend et al., 2006). The common pitfall for each of these methods is that detection relies upon primers that are designed from existing sequences, which presumes that the unknown virus will resemble previously sequenced viruses. In addition, with the exception of complete genomic sequencing, most of these methods characterize small genomic regions, thus offering a partial view of the virus.

Another issue that affects the rapid and precise NDV characterization is the frequent occurrence of mixed NDV infections in birds with viruses of different genotypes. In these cases, any primer-specific method will amplify the viral product most homologous to the primer(s) of choice, but not necessarily the most abundant or representative of the viral population infecting the birds. Random genome sequencing represents an unbiased and thorough alternative that has been widely used to characterize the genomes of large DNA viruses (Afonso et al., 2006), but has only recently been used with NDV (Djikeng et al., 2008). A protocol that follows two basic principles: (i) random amplification of total RNA, and (ii) random selection of colonies followed by sequencing and assembly has been developed.

This protocol, a modification of the sequence-independent, single-primer amplification, SISPA method, first developed by Reyes and Kim (Reyes and Kim, 1991), and recently adapted for NDV entails the directional ligation of an asymmetric primer at either end of a blunt-ended DNA molecule (Djikeng et al., 2008). Following several cycles of denaturation, annealing and amplification, minute amounts of the initial cDNA are enriched and then cloned, sequenced and analyzed. Several modifications of the SISPA method have so far been implemented including random-PCR (rPCR) (Froussard, 1992). The rPCR method combines reverse transcription primed with an oligonucleotide made up of random hexamers tagged with a known sequence, which is subsequently used as a primer-binding extension sequence. This initial modification was first used to construct a whole cDNA library from low amounts of viral RNA.

A more recent modification, the DNAse-SISPA technique (Allander et al., 2001; Allander et al., 2005; Breitbart and Rohwer, 2005), includes steps to detect both RNA and DNA sequences. The combining of sample filtration through a 0.22 μm column and a DNAse I digestion step led to the identification of viruses from clinical samples. In addition, to its utility for viral discovery, the DNase-SISPA method has utility in obtaining full genome sequence from uncharacterized viral isolates or viral isolates from highly divergent families. The SISPA method was used as a rapid and cost effective method for generating full genome coverage of NDV. A total of 349 sequences were needed to completely sequence the LaSota NDV genome, with a redundancy of 13.72. A limitation to the method includes the need for samples that contain a large number of viral particles (10^6 particles in 0.2 ml samples). Moreover, because the capsid structure renders the viral genomes nuclease-resistant, this protocol requires encapsidated viral genomes to allow the removal of most extra-viral contaminants. For samples with high levels of host nucleic acid contamination, we have used 5 μg of RNAse A to treat 500 μl of filtered virus for 1 hr. We have found that RNAse A treatment eliminates the majority of host RNA derived sequence contaminant in these cases (Afonso et al., unpublished observations). In addition, DNAse I can be used to reduce host contaminants (Djikeng et al., 2008).

7. Conclusions

Because of the highly contagious nature of NDV and its clinical similarity to highly pathogenic avian influenza, accurate monitoring and rapid diagnosis of bird infections are crucial to any control and eradication program. Active surveillance of wild birds, LBM, and poultry production sites should increase our understanding of the predominance and evolution of NDV. Although surveillance should continue in some recently found reservoirs of NDV (including waterfowl and shorebirds), the search for additional natural reservoirs of virulent viruses should continue. Live bird markets are specifically significant because they promote the commingling of multiple bird species in close quarters and provide an environment in which reservoir species such as waterfowl are closely housed with gallinaceous hosts. Many challenges still remain on the detection of vNDV. Prompt detection and differentiation is challenging due to the broad genetic diversity of NDV and because vaccine and endemic viruses are often serologically indistinguishable. Rapid differentiation of loNDV strains from vaccines and identification of new forms of vNDV needs to be investigated further. In addition, phylogenetic analysis reveals that loNDV and vNDV are continually evolving. For improved surveillance and to increase our understanding of the ecology of NDV among wild bird and domestic poultry populations, specific diagnostic assays capable of identifying the full range of circulating NDV genotypes are needed. Similar to the situation with diagnostic assays for highly pathogenic avian influenza (HPAI), continual optimization must be carried out to ensure that all NDV are detected, whether they are from a wild bird species or from poultry. While continued genetic characterization of circulating strains by traditional methods is likely to provide evolutionary information and to help expand the arsenal of PCR based detection methods, random sequencing should be incorporated as a tool to identify new genotypes. Finally, consideration to include objective and uniform criteria to classify NDV into genotypes should be made.

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